Loss of Bacterial Diversity During Antibiotic Treatment of Intubated Patients Colonized with *Pseudomonas aeruginosa*.

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Conflicts of interest:

Summary

Background

Managing airway infections caused by *Pseudomonas aeruginosa* is a serious clinical challenge but little is known about the dynamics of bacterial communities within the context of *P. aeruginosa* infection of intubated patients. In this study, bacterial community composition of lung lavage and endotracheal aspirates from intubated patients who developed acute *Pseudomonas aeruginosa* infection were analyzed using culture-independent methods.

Methods

16S ribosomal RNA (rRNA) clone libraries and microarrays (PhyloChip) were used to determine changes in bacterial diversity over time in six patients with hospital-acquired *P. aeruginosa* and a single patient chronically colonized with *P. aeruginosa*. Three additional patients, briefly intubated during elective surgery, were also evaluated as controls.

Findings

Bacterial community analyses demonstrated absence of bacterial DNA in the endotracheal aspirates of briefly intubated control patients. However, bacterial 16s rRNA genes were readily detected in endotracheal aspirates and bronchoalveolar lavage samples from patients intubated for longer periods. . Bacterial composition of paired endotracheal aspirate and bronchoalveolar lavage samples from individual patients were comparable. Clone library and microarray analysis of the 16S rRNA amplicon pool demonstrated the presence of oral-, nasal- and gastrointestinal-associated bacteria, including known pathogens, in all patients colonized with *P. aeruginosa*. However, the PhyloChip also

detected many additional bacterial groups undetected by clone libraries. Both culture-independent methods demonstrated that bacterial diversity decreased following antibiotic administration and the majority of communities became dominated by *P. aeruginosa*. Specifically, administration of anti-*Pseudomonal* antibiotics appeared to increase the relative abundance of *P. aeruginosa*. PhyloChip data also demonstrated a pattern of reciprocal abundance between phylogenetically distinct bacterial groups, suggesting that these groups compete for a similar ecological niche.

Interpretation

Culture-independent analysis of lung fluid from intubated patients revealed the presence of an enormous diversity of oral and gut flora that are aspirated around the endotracheal tube. Our study of patients selected only for the presence of *P. aeruginosa* shows that antimicrobial therapy in general, and anti-*Pseudomonal* antibiotics in specific, leads to a dramatic loss of microbial diversity and selection for P. aeruginosa or other pathogens and was associated with poor outcomes.

Introduction

Mechanically ventilated patients develop bacterial colonization of the oropharynx and endotracheal tube within twelve hours of intubation.[1,2] During ventilation the endotracheal tube decreases normal airway defenses and allows microbe-laden oropharyngeal or gastric secretions to be aspirated around the endotracheal tube cuff into the lower airways.[1] The fate of such bacteria in the lungs of intubated patients is largely unknown. To date, investigations of intubated patients have used aerobic cultures of either endotracheal secretions or bronchoalveolar lavage specimens to determine the

presence of pathogenic bacteria. However, these techniques neither determine the dominant bacterial species nor the range of bacterial diversity within the community. Culture-independent methods provide a comprehensive view of bacterial diversity and may be used to evaluate complex bacterial community dynamics, particularly during antibiotic therapy.

Culture-independent analyses are typically based on biomarker identification. The 16S rRNA gene is the most commonly used biomarker for bacterial community studies.[3] Highly conserved regions of the 16S rRNA gene enable amplification of this gene from most bacteria using "universal" PCR primers, while variable regions within this gene permit discrimination between bacterial types.[4] This approach has been applied successfully to the analysis of environmental[5-9] and human[10-14] bacterial communities, and has revealed a much broader bacterial diversity than traditional culture-based techniques.[3] However, to our knowledge, no previous study has examined the true extent of bacterial diversity within the lungs of intubated patients using culture-independent methods.

Here, we report the use of two 16S rRNA gene-based culture-independent methods, clone library sequencing (the current gold-standard in microbial ecology)[15] [milestone reference] and a novel high-density oligonucleotide microarray (PhyloChip).[16,17] [incorrect refs – these ref old version of chip – use Brodie et al 2006]. Clone libraries typically involve sequencing a few hundred 16S rRNA genes following PCR amplification and as such only profile the dominant organisms of the bacterial population. Where one organism predominates, less abundant species that may contribute to disease pathogenesis, may remain undetected. For this reason, a novel microarray approach was

applied to identical samples. The advantage of the microarray is its capacity to analyze entire pools of PCR products simultaneously, permitting detection of many more species present including those of lower abundance.

The aim of this study was to use culture-independent methods to determine changes in bacterial community composition of the lungs of intubated patients with hospital-acquired *P. aeruginosa* during antimicrobial treatment.

Methods

Patient selection and sampling protocol

Daily endotracheal aspirates (EA) were collected from all intubated patients in the medical, surgical, neurovascular and cardiac intensive care units at the University of California San Francisco (UCSF). Daily EA samples were obtained and all samples were screened by culture on Difco *Pseudomonas*-isolation agar (Becton Dickinson, NJ). *Pseudomonas* positive patients were approached, gave informed consent and daily quantitative cultures of *P. aeruginosa* were performed on their respiratory samples. Extensive clinical data were collected so severity of illness could be correlated with 16S rRNA data. Antibiotic drug history was also recorded. All protocols were approved by the Committee on Human Research at UCSF. Using a CombiCath catheter, blind mini-bronchoalveolar lavage (blind mini-BAL) was performed when clinical infection was suspected. The right bronchoalveolar tree was irrigated with 3 x 20 mL aliquots of non-bacteriostatic saline. The resulting lavage was used for *P. aeruginosa* culture and 16S rRNA analysis. Blind mini-BALs and EAs were centrifuged and stored at -80 °C.

DNA Extraction and amplification of bacterial 16S rRNA gene.

Bacterial genomic DNA was isolated from 0.5 ml of EAs and BALs using Promega (Carlsbad, CA) Wizard Genomic DNA purification Kit according to manufacturer's instructions for purification of both Gram negative and Gram positive bacteria. The 16S rRNA genes were amplified from extracted DNA using universal bacterial primers Bact-27F (5'-AGAGTTTGATCCTGGCTCAG-3') and Bact-1492R (5'-GGTTACCTTGTTACGA CTT-3').[18] The reaction mixture (50 μl final volume) contained 5 μl 10x PCR buffer (Amersham, NJ), 1 μl dNTPs (10mM), 0.7 μl forward primer and reverse primer (100pmol/ul each), 0.35 μl Taq polymerase (5 U/μl; Amersham, NJ) and 1 μl of template DNA. PCR was performed using the DNA Engine Tetrad thermal cycler (Bio-Rad, CA). To maximize the number of bacterial species that could be recovered by PCR three different annealing temperatures (48 °C, 52 °C and 56 °C) were used for each sample to amplify 16S rRNA genes. The following cycling parameters were used: 3 min of initial denaturation at 95 °C followed by 25 cycles of denaturation (30 s at 95 °C), annealing (30 s), and elongation (120 s at 72 °C), with a final extension at 72 °C for 7 min. Amplified products from all samples were verified by gel electrophoresis. All PCR products were gel purified using the QIAquick gel extraction kit (Qiagen, CA), and for each sample, the purified products amplified using three different annealing temperatures were pooled together for cloning and sequencing and microarray analysis.

Cloning and sequencing

To generate libraries for each sample the respective PCR products were cloned into pCR4-TOPO vectors (Invitrogen, CA) according to the manufacturer's instructions. One hundred and ninety two transformants from each library were picked randomly. Double-ended sequencing reactions were carried out using PE BigDye terminator chemistry (Perkin Elmer, MA) and resolved using an ABI PRISM 3730 (Applied Biosystems, CA) capillary DNA sequencer. Sequencing was performed at the DOE Joint Genome Institute (JGI).

Sequence alignment and phylogenetic analysis

Individual sequencing reads were assembled using Phred and Phrap[19,20] and were required to pass quality tests of Phred 20 (base call error probability < $10^{-2.0}$) to be included in analysis. An online tool at Greengenes [greengenes ref here, DeSantis 2006 – AEM) (http://greengenes.lbl.gov) was used to detect putative chimeric sequences using an updated version of Bellerophon[21]. Sequences were aligned to the Greengenes 7,682-character format using the NAST[22] web-server prior to being assigned to a taxonomic node using a sliding scale of similarity thresholds[23] using the Greengenes classify tool. Distance matrices were constructed for each library using the distance matrix tool at Greengenes with NAST aligned sequence data as input.

Phylotype clustering and diversity estimates

Using the distance matrices generated, numbers of 16S rRNA gene phylotypes were calculated at 99% homology using furthest neighbor clustering in the program DOTUR (ref Schloss) with 1000 iterations for bootstrapping. A representative 16S rRNA gene-

based phylogenetic tree was constructed in the software package ARB [24] using data from the Greengenes database.

DNA Sequence Accession numbers

Sequences generated in this study have been deposited in GenBank under accession numbers XXXXX-XXXXX.

PhyloChip Processing, Scanning, Probe Set Scoring and Normalization.

The pooled PCR product was spiked with known concentrations of synthetic 16S rRNA gene fragments and non-16S rRNA gene fragments as internal standards for normalization with quantities ranging from 5.02×10^8 and 7.29×10^{10} molecules applied to the final hybridization mix. Target fragmentation, biotin labeling, PhyloChip (Affymetrix??) hybridization, scanning and staining were as described by Brodie et al (2006 ref), while background subtraction, noise calculation, and detection and quantification criteria were essentially as reported in Brodie et al (2006 ref), with some minor exceptions. For a probe pair to be considered positive, the difference in intensity between the perfect match (PM) and mismatch (MM) probes must be at least 130 times the squared noise value (N). A taxon was considered present in the sample when 90 % or more of its assigned probe pairs for its corresponding probe set were positive (positive fraction, pf >= 0.90). Hybridization intensity (referred to as intensity) was calculated in arbitrary units (a.u.) for each probe set as the trimmed average (maximum and minimum values removed before averaging) of the PM minus MM intensity differences across the

probe pairs in a given probe set. All intensities < 1 were shifted to 1 to avoid errors in subsequent logarithmic transformations. To account for scanning intensity variation from array to array, the intensities resulting from the internal standard probe sets were natural log transformed. Adjustment factors for each PhyloChip were calculated by fitting a linear model using the least-squares method. A PhyloChip's adjustment factor was subtracted from each probe set's ln(intensity). Intensities for patient 1049 were also normalized by total array intensity. When summarizing PhyloChip results to the sub-family, the taxon with a probe set producing the highest intensity within a sub-family was used.

Results

We began by comparing sampling techniques. To do this, bacterial diversity of samples obtained using mini blind-BAL were compared to those obtained using endotracheal aspirates from two patients with hospital-acquired *P. aeruginosa* using 16S rRNA clone library sequencing. While there were distinct differences in community composition between individual patients, community composition of EAs and BALs from the same individual were highly similar (Figure 1A and B). For the remainder of this study, EAs were used due to the simplicity and cost effectiveness of this less invasive sample collection method.

Next, as a control for this study, EA samples were collected from three normal individuals briefly intubated for elective surgery. No 16S rRNA PCR product was detected in these patients (data not shown) using conditions that readily yielded 16S rRNA amplicons in study patients, confirming that the normal lung is sterile and that our

techniques identify organisms present only after colonization of the endotracheal tube or airway has occurred.

Figure 2 details age, sex, date of initial ventilation, dates of EA sampling, periods of anti-*Pseudomonal* antibiotic administration and patient outcomes for all study patients. All patient EA samples yielded a 16S rRNA PCR product and following cloning a total of 3,278 nonchimeric 16S rRNA sequences were subjected to phylogenetic analysis. Almost all organisms detected by cloning were from five bacterial phyla, the *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria* and *Fusobacteria* (Figure 3). Over half (55%) of the sequences obtained were from *Pseudomonas aeruginosa*, followed by *Stenotrophomonas maltophila* (9.7%), *Prevotella* spp. (5.8%), *Acinetobacter* spp. (5.7%), *Serratia marcescens* (5.0%), *Haemophilus* spp. (3.8%), *Neisseria* spp. (3.3%), *Mycoplasma* spp. (2.4%) and *Streptococcus* spp. (2.3%). An additional 18 genera were also detected but together represented less than 7% of all clones sequenced. Of these less abundant species detected, many are known oral, nasal and gastrointestinal tract inhabitants e.g. *Porphyromonas, Campylobacter, Fusobacter, Lactobacillus, Enterococcus, Rothia, Actinomyces, Abiotrophia, Alcaligenes, Corynebacterium, Staphylococcus* and *Veillonella* refs.[26,27] J Clin Microbiol. 2005 Feb;43(2):843-9. These results support the hypothesis that oral, nasal and gastrointestinal tract microbiota are the major reservoirs for bacteria that colonize the lower airway in intubated patients.[29,30]

We evaluated bacterial diversity in 5 patients (1049, 1150, 1900, 1578 and 1523) who had an initial sample obtained before or within 24 hours of parenteral antibiotic

administraton and a second sample obtained 4 to 10 days later. Analysis of microbial diversity by 16S clone library demonstrated a substantial reduction in bacterial diversity during periods of antimicrobial administration (Figure 4), with the mean number of bacterial species identified falling from **NN to N.** The net result of antibiotic therapy was the selection of a few species that dominate the community. Significantly, despite administration of anti-*Pseudomonal* therapy, bacterial communities from four of these 5 patients became dominated by *P. aeruginosa,* and the fifth also became dominated by a pulmonary pathogen (*Klebsiella pneumoniae*).

We obtained additional samples at later timepoints in two of these patients (1150 and 1523), and in two additional patients we obtained initial EA samples during anti-Pseudomonal therapy and follow-up samples after completion of the antibiotic course (901 and 151). Collectively, these data suggest that bacterial diversity and Psuedomonas dominance are highly correlated during anti-Pseudomonal therapy. All of these samples showed reduced diversity and in 6 of 9, *Pseudomonas aeruginosa* was the predominant species suggesting that once this organism is established as the dominant species, microbial diversity is slow to recover. Interestingly

Dominance of bacterial communities by one or a few species may result either from overgrowth of the dominant species or loss of the non-dominant species. Due to the limited numbers of clones that can feasibly be sampled from clone libraries, highly abundant species may mask the presence of less abundant, but clinically significant bacteria. To determine whether the decline in diversity seen in clone libraries was a true reflection of the bacterial community in these patients, we also analyzed bacterial

diversity using high-density microarrays (PhyloChips) which have enhanced sensitivity to lower abundance species when compared with cloning, but less species specificity. (DeSantis, Microbial Ecology, in press). For four patients, the same 16S rRNA gene amplicon pools from which clone libraries were prepared were subsequently hybridized to PhyloChips and the bacterial communities compared. While the microarray approach detected orders of magnitude more bacterial types than cloning, it is clear that the patterns of changing diversity in patients over time are comparable between these two culture-independent methods (Figure 5). Due to the increased sensitivity of the PhyloChip, entire bacterial community responses can be monitored. Figure 6a illustrates temporal changes in fluorescence intensity of bacteria detected by PhyloChip. Bacteria demonstrating large changes in intensity between time points are labeled and correspond well to the dominant bacteria detected by clone library analysis. The dominance of a few species within a community resulted in the inability of cloning to detect less abundant bacteria. However, the PhyloChip demonstrated that many of the bacteria present at the initial sampling point were indeed still present at the subsequent sampling period (Figure 6b). Conversely, bacteria such as *Klebsiella* that became dominant in later samples were detected in the initial sample by PhyloChip, but were not detected by cloning. This underscores the potential for initially low abundant species to eventually dominate bacterial communities during the course of antimicrobial administration.

A notable trend is the phylogenetic specificity of the bacterial response over time; entire groups of bacteria tend to respond in a similar manner. For example the γ-Proteobacteria (which includes *Pseudomonas aeruginosa*) generally exhibit an inverse relationship in

abundance with bacteria in the phylum *Actinobacteria* and in the class *Bacilli* (which includes *Lactobacillus, Streptococcus, Staphylococcus* and *Enterococcus*) in all patients examined (Figure 6a). Similarly *Haemophilus* and *Pseudomonas* also demonstrated an inverse relationship. The reciprocal changes in these subgroups suggest they are competing for a similar niche in the bacterial community.

Discussion

Managing infections caused by *P. aeruginosa* is increasingly difficult due to this bacteria's metabolic versatility, intrinsic antimicrobial resistance and its remarkable armory of virulence factors. Clinically, treatment options are becoming limited due to the rapid emergence of multidrug-resistant strains, which are now estimated to account for up to 30% of strains isolated from patients in nursing homes, hospitals and intensive care units.[31] ray ref

In this study, we used 16S rRNA-based culture-independent methods to determine the effects of anti-*Pseudomonal* therapy on bacterial community dynamics in patients with hospital-acquired *Pseudomonas aeruginosa*. Compared to current clinical culture methods, both clone library and microarray techniques have often provided a richer picture of microbial diversity [25,26,32], and we found that to be true in this study. Prior to, or early in antibiotic therapy, the airways are colonized with a remarkably wide array of oral, nasal and gut flora that are presumably aspirated into the lung around the endotracheal tube. Not unexpectedly, antimicrobial treatment has a pronounced effect on bacterial community composition with bacterial diversity falling in every case. Alarmingly, pathogenic species became dominate in every patient during anti-

Peudomonal therapy and both the loss of diversity and *Pseudomonas* dominance persisted long after antibiotic therapy. These findings document the frequent failure of antimicrobial therapy to eradicate pathogenic species from the airways in intubated patients in the ICU setting and suggest that the loss of microbial diversity and pathogenicity may be linked.

It has long been hypothesized that the evolution of virulence is related to the number and variety of bacterial species infecting the host.[35-37] Previously, it was assumed that increased diversity of pathogenic species would promote virulence of individual species.[35] However, more recently it has been demonstrated that in mixed bacterial populations, less virulent strains are often favored, suggesting that increased diversity may reduce virulence.[38] This hypothesis is supported by our observations that administration of antimicrobials eliminates competition by decreasing diversity of non-target organisms coincident with an increase in pathogen abundance. One mechanism through which the dramatic decrease in bacterial diversity might alter virunence is through quorum sensing. Quorum sensing is means of bacterial cross-talk between individual cells incorporated into a biofilm and it may radically affect gene expression and virulence of pathogens, including *Pseudomonas aeruginosa*. Further studies investigating the contribution of bacterial dynamics to pathogenicity will be required to fully evaluate this hypothesis.

While the PhyloChip was found to be superior to clone library sequencing for assessment of clinical samples due to its ability to detect all bacteria, including low abundance species, clone library approaches retain utility by providing important information on bacterial relative abundance. These culture-independent molecular methods documented a multitude of bacteria undetected by standard identification techniques. Importantly, this was true not only for fastidious, slow-growing, and/or nonculturable microorganisms but also for routinely cultured pathogens.[3] These data highlight the inadequacies (extended processing time and limited information) of traditional culture-based detection that may result in sub-optimal therapeutic decisions. Application, therefore, of the PhyloChip in a clinical setting has the potential for improved patient care.

This study was limited to 7 patients and carried out as a proof of principle investigation. Since the focus of this research was patients culture-positive for *P. aeruginosa*, the application of 16S rRNA molecular detection techniques will be expanded to other patient groups pre- and post-antimicrobial administration. Future research using molecular monitoring of the bacterial communities in intubated patients may ultimately aid in the creation of patient-tailored therapies to help reduce the proliferation of pathogens such as *P. aeruginosa*, thus curbing virulence and improving patient outcome.

[Discussion is too short as it stands, what else should we include? None of the points on broad-spectrum antimicrobial use were included as they seemed inconsistent and it's

asking for trouble if we draw conclusions based on one or two patients. Bibliography will be completed on Monday]

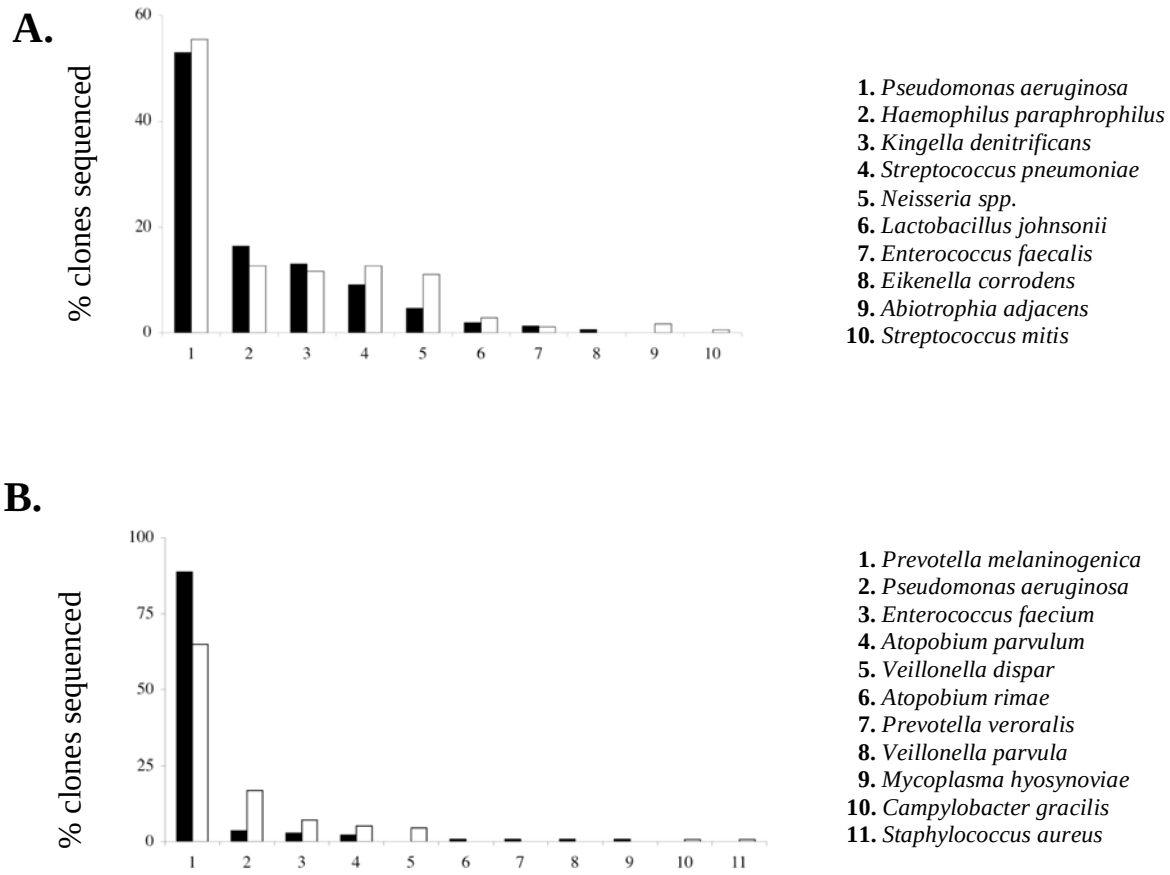

Figure 1. Comparison of 16S rRNA clone libraries of EA (white bars) and BAL (black bars) patient samples. A. Patient 1523; **B.** 1150 Percentage of clones sequenced from each library are comparable.

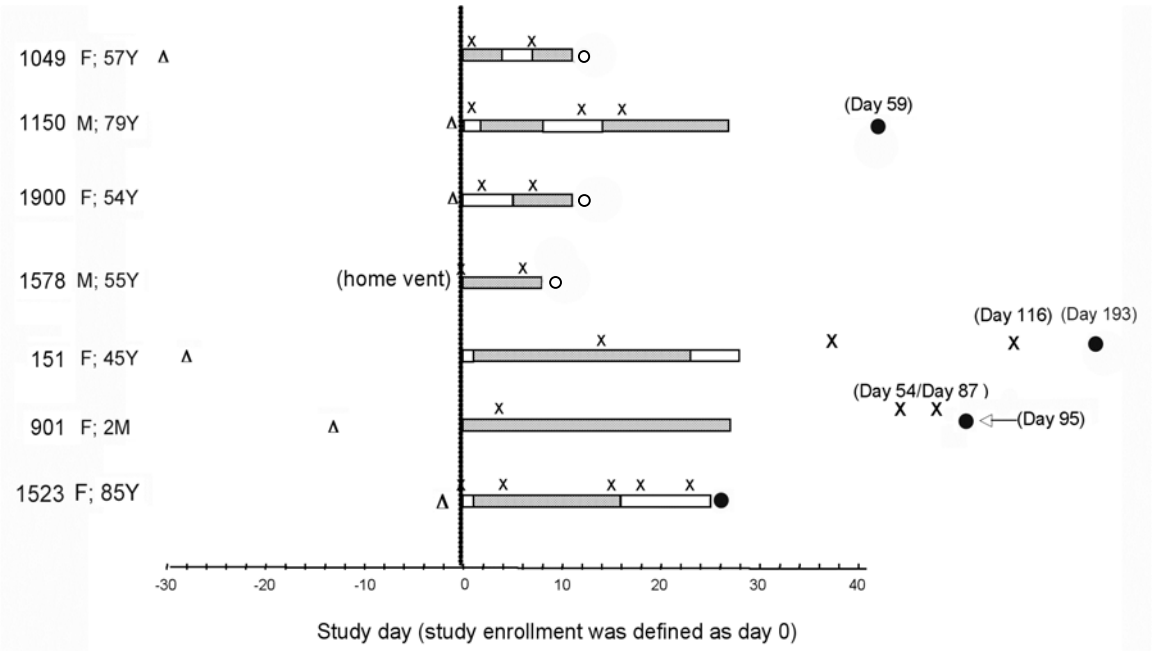

Figure 2. **Patient data showing date of mechanical ventilation, date of sputum collection, antibiotic status and patient survival.** Patient codes are shown on left hand side of plot. Patient sex (M, male; F, female); Age (Y, years; M, months); Δ Initial date of mechanical ventilation; X Sputum samples collected; ● Patient expired; ○, Patient alive at ICU discharge; □, Without antipseudomonal-antibiotics coverage; ▨, With antipseudomonal-antibiotics coverage.

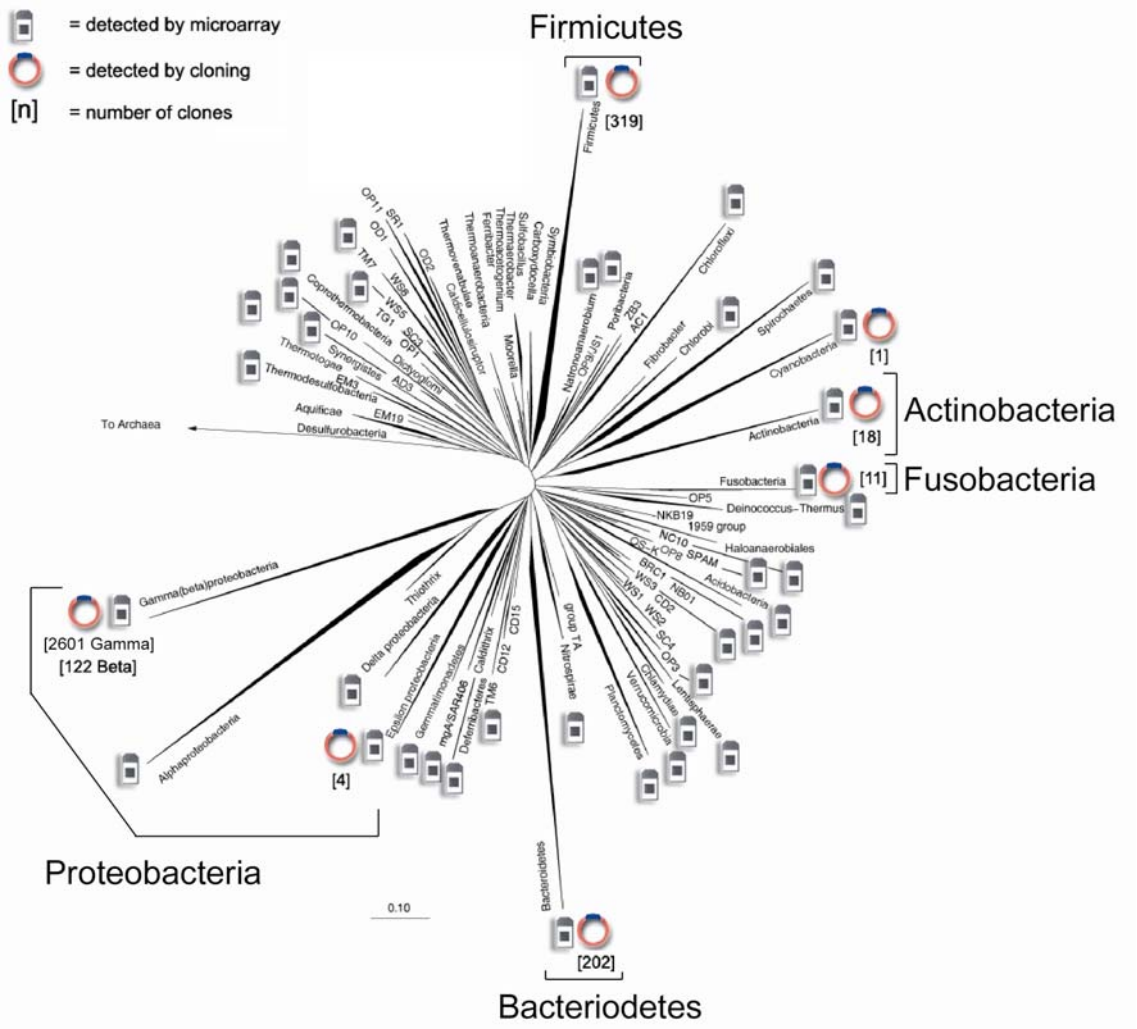

Figure 3. Representative phylogenetic tree showing all recognized bacterial phyla/divisions. Phyla detected in endotracheal aspirates by both cloning and PhyloChip microarray analyses are shown. The five main phyla detected by clone library are indicated. Many additional phyla, undetected by cloning, were detected by array analysis.

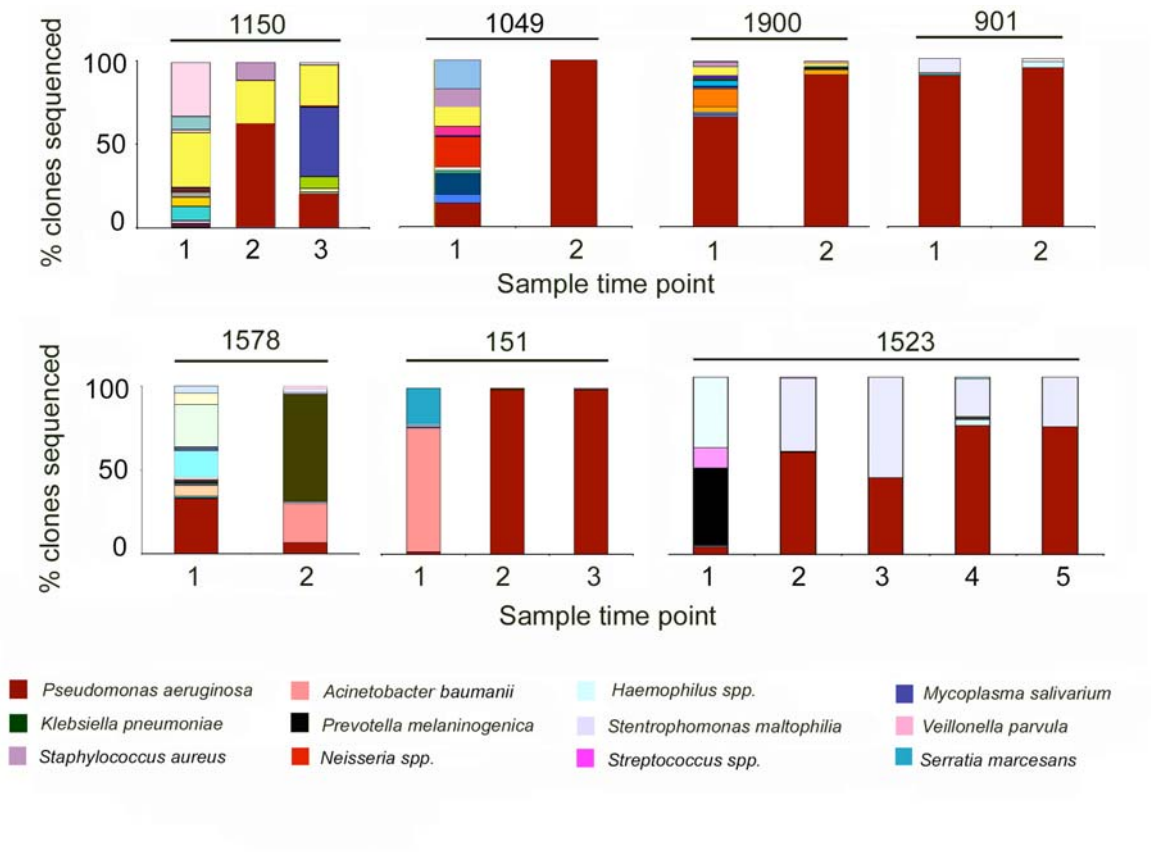

Figure 4. Temporal changes in bacterial diversity. Proportional changes in bacterial distribution in endotracheal aspirates over time as determined by clone library analysis. Numbers in bold above plots represent patient codes. [change Prevotella legend box color – should be yellow]

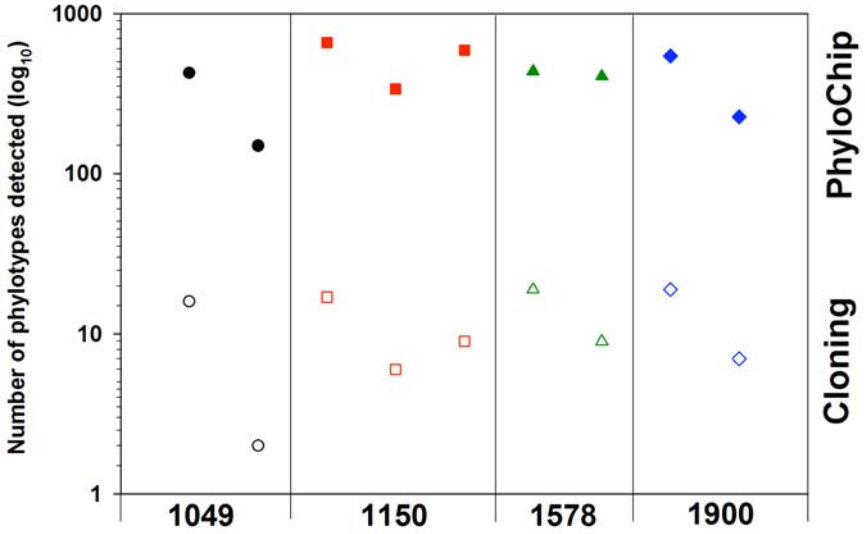

Figure 5. Comparison of PhyloChip and clone library monitoring of bacterial diversity (phylotype numbers) over time for four patients. Closed symbols show number of bacterial phylotypes detected by PhyloChip analysis, open symbols show number of bacterial phylotypes detected by clone library.

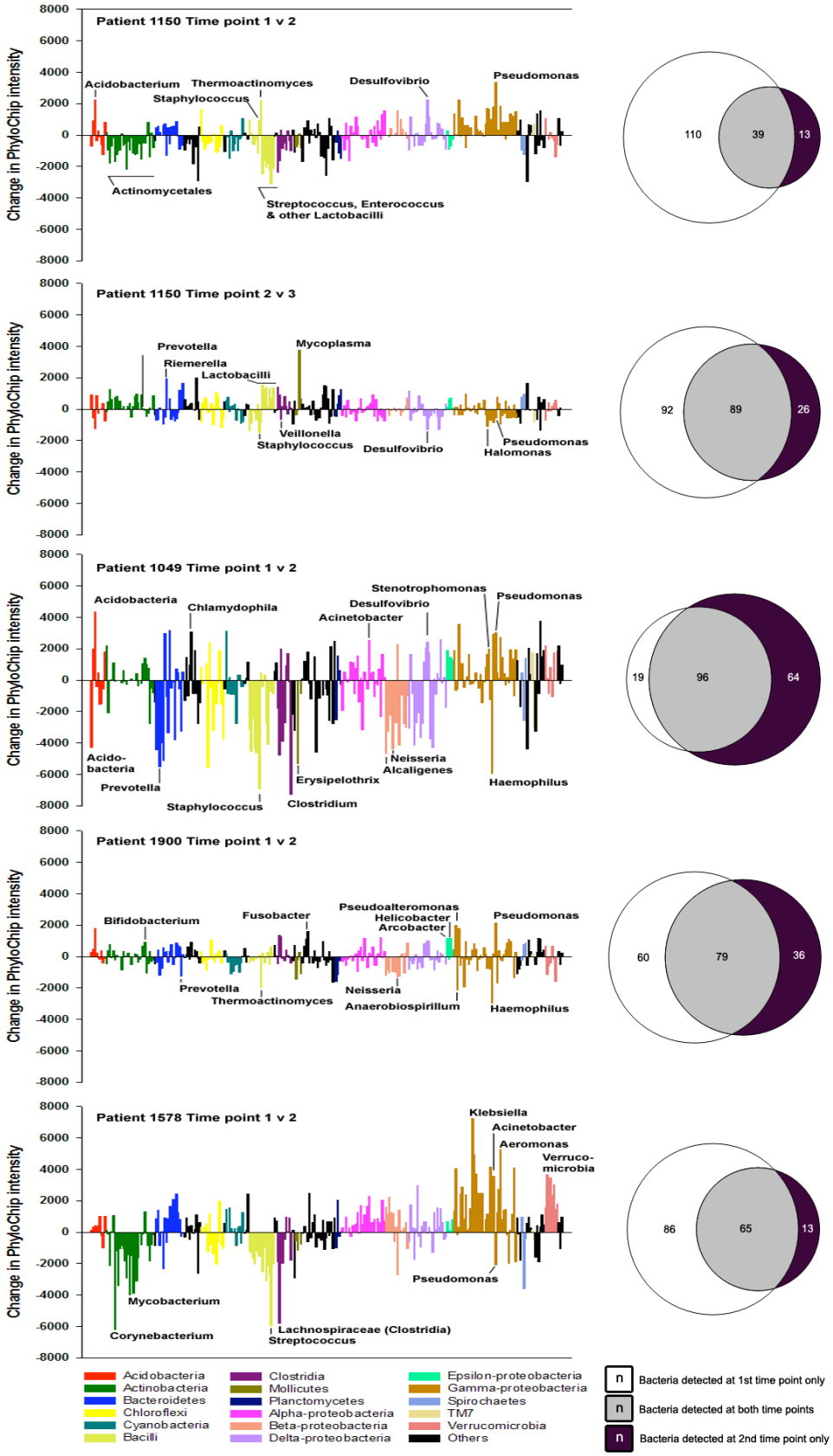
Patient 1150 Time point 1 v 2
Change in PhyloChip intensity
Acidobacterium
Thermoactinomyces
Staphylococcus
Desulfovibrio
Pseudomonas
Actinomycetales
Streptococcus, Enterococcus & other Lactobacilli
110
39
13
Patient 1150 Time point 2 v 3
Prevotella
Riemerella
Lactobacilli
Mycoplasma
Veillonella
Staphylococcus
Desulfovibrio
Pseudomonas
Halomonas
92
89
26
Patient 1049 Time point 1 v 2
Acidobacteria
Chlamydophila
Desulfovibrio
Acinetobacter
Stenotrophomonas
Pseudomonas
Acido-bacteria
Prevotella
Staphylococcus
Erysipelothrix
Clostridium
Neisseria
Alcaligenes
Haemophilus
19
96
64
Patient 1900 Time point 1 v 2
Bifidobacterium
Fusobacter
Pseudoalteromonas
Helicobacter
Arcobacter
Pseudomonas
Prevotella
Thermoactinomyces
Neisseria
Anaerobiospirillum
Haemophilus
60
79
36
Patient 1578 Time point 1 v 2
Klebsiella
Acinetobacter
Aeromonas
Verruco-microbia
Mycobacterium
Corynebacterium
Lachnospiraceae (Clostridia)
Streptococcus
Pseudomonas
86
65
13
Acidobacteria
Actinobacteria
Bacteroidetes
Chloroflexi
Cyanobacteria
Bacilli
Clostridia
Mollicutes
Planctomycetes
Alpha-proteobacteria
Beta-proteobacteria
Delta-proteobacteria
Epsilon-proteobacteria
Gamma-proteobacteria
Spirochaetes
TM7
Verrucomicrobia
Others
n
Bacteria detected at 1st time point only
Bacteria detected at both time points
Bacteria detected at 2nd time point only

Figure 6. PhyloChip analysis of complete bacterial communities over time in endotracheal aspirates. (A) Bacteria are ordered alphabetically from left to right according to taxonomic affiliation. Bars above the zero line represent bacteria which increased in abundance relative to the first EA sample compared, bars below represent those bacteria that declined in abundance. **(B)** Venn digrams to the right demonstrate the number of bacterial subfamilies detected at each time point and the intersection in composition between time points.

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